REMARKS

Claims 16, 26-33, 37, 38, and 40-50 were pending in this application. Claims 41-50 are withdrawn pending rejoinder if product claims are allowed. Claims 34-36 and 39 are cancelled herein, and claims 16, 32, 38, and 40 are amended. Claims 16, 38 and 40 are amended to recite that the claimed polypeptides bind to WSX-1/TCCR. Claim 38 is further amended to recite that the claimed polypeptide comprises an amino acid at least 90% identical to a particular sequence. New claims 51-57 are added herein. Applicants acknowledge that the Examiner states that claim 32 would be allowable if drafted in independent form. Thus, claim 32 is amended to independent form. The specification is amended to indicate the status of a priority application, which has issued as a patent, and for grammatical consistency.

Restriction Requirement

The Applicants take note of the finality of the restriction requirement but would like to point out again that no serious burden was found in examining SEQ ID NO:2 and SEQ ID NO:6 together in the parent case (U.S. Ser. No. 10/000,776). The similarity of these sequences is illustrated in Fig. 1, in which the sequences are shown to be identical in all but the first 10 and 11 amino acids respectively. These sequences are identical for 232 amino acids out of 242 and 243 amino acids respectively, *i.e.*, for greater than 95% of their lengths. Searching the sequence of 232 identical amino acids would provide an overlapping set of reference with no undue burden. That there is no burden would appear to be particularly true since no prior art rejections have been made in the first office action. Further, the ease with which similar sequences can be identified is illustrated by the identification of Sheppard et al., U.S. 6,822,082 on page 7 of the office action. Given these observations, the Applicants request reconsideration of the finality of the restriction requirement and ask that the search be expanded to include at least SEQ ID NO:2 and SEQ ID NO:6.

35 U.S.C. § 112, ¶ 2

Claims 34-36 are rejected under 35 U.S.C. § 112, ¶2. Applicants disagree that Claim 34 is indefinite because it recites "a cell surface receptor, " (see, for example, *Suzuki et al.*, Proc. Natl. Acad. Sci. USA, 79:591-595, 591 (1982) (enclosed) ("A cell surface receptor is defined as a molecule that transmits, upon specific binding of ligand, a signal that affects cell functions.") and *Principles and Techniques of Practical Biochemistry* 403-405 (Keith Wilson & John Walker eds., 5th Ed., Cambridge Press 2000) (which teaches the structure of a cell surface receptor). Nonetheless, claims 34-36 have been cancelled herein for other reasons and the rejection is moot.

With respect to claim 36, the Examiner indicates that the term "WSX-1/TCCR" is a arbitrary name that is not recognized in the art. Although claim 36 is cancelled herein, claims 16, 38, and 40, as amended, include the term. The WSX-1/TCCR (T-cell cytokine receptor) is well known as evidenced by *Yoshida et al.*, Immunity, 15:569-578 (2001) (page 569 second full paragraph, right column) (enclosed), which references *Sprecher et al.*, Biochem. Biophys. Res. Commun., 246:82090 (1998)) and *Chen et al.*, Nature, 407:916-920 (2000) as teaching WSX-1/TCCR. Furthermore, the specification defines what is meant by "WSX-1/TCCR" on page 9, lines 26-27, by providing a reference, a Gen Bank accession number, and two sequences. Any one of these would have been sufficient to define the term "WSX-1/TCCR," and newly amended claims 16, 38, and 40 should not be rejected under 35 U.S.C. § 112, ¶ 2.

35 U.S.C. § 112, ¶ 1

Claims 16, 26-31, and 33-40 are rejected under 35 U.S.C. § 112, ¶ 1. The Office Action states that the specification allegedly fails to enable claims to an isolated polypeptide comprising at least 17, 20, 25, 30, 35, 50, or 75 amino acids of SEQ ID NO:2 or % variants of SEQ ID NO:2 because the claims do not require that said polypeptides possess any particular function. Claims 16, 38, 40 have been amended to recite that the claimed polypeptides bind to WSX-1/TCCR, and claims 34-36 are cancelled herein. As claims 26-31 and 33 are dependent on claim 16, Applicants believe this amendment fully overcomes the rejection.

Applicants respectfully point out that the test for enablement is whether experimentation alleged to be necessary is undue, *not whether any experimentation is necessary. In re Angstadt*, 537 F.2d 498, 504 (CCPA 1976). When the art typically engages in a type of experimentation, that experimentation is not considered undue. *In re Wands*, 858 F.2d at 737. A patent is not required to teach what is well-known in the art, rather such information is preferably omitted. *In re Buchner*, 929 F.2d 660, 661 (Fed. Cir. 1991).

It is easily within the skill of a skilled artisan to generate (*see, e.g.*, p. 31, lines 10-16) and identify (*see, e.g.*, p. 33-36 and 54 (line 12)-55 (line 9)) the claimed fragments with the recited functionality without undue experimentation. Furthermore, the Office Action states that the specification teaches methods to detect receptor binding (Office Action, page 5). The number of fragments covered by amended claim 16 is reasonable given the ease with which one of skill in the art can identify and create the fragments, especially as the sequence will be contained within the sequence defined by SEQ ID NO:2, given the teaching the specification as to how binding to WSX-1/TCCR can be detected. Thus, identification of the fragments defined in amended claims 16, 38, and 40 and claims dependent on claim 16 will not require undue experimentation, but rather such identification is within the routine arsenal of one of skill in the art, given the teachings of the specification. Applicants respectfully request reconsideration and withdrawal of the enablement rejection.

Claims 16, 26-31, and 33-38 and 40 are further rejected under 35 U.S.C. §112, ¶1, as allegedly lacking sufficient written description because they fail to specify a particular biological function for the claimed polypeptides. As described above, claims 16, 38 and 40 are amended herein to specify binding to WSX-1/TCCR. As noted at MPEP 2163 II.A. (Rev. 5), "[t]here is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed." Furthermore, applicants respectfully point out that Example 14 of the Written Description Guidelines indicates that variants of a single disclosed species claimed with a functional limitation has sufficient written description where the procedures for making

variants are known and where an assay is described for identifying the presence of a claimed function.

As amended, claims 16, 38, and 40, as well as claims dependent on claim 16, are directed to polypeptides that bind to WSX-1/TCCR. The fragments and variants are described at least at page 11 (lines 1-7 and lines 14-22) and page 13 (line 4) – page 19 (line 10). As described above, assays for identifying binding to WSX-1/TCCR are provided in the specification as well. Given the amendment of the claims and the teachings of the specification, Applicants request reconsideration and withdrawal of the rejection under 35 U.S.C. § 112 for lack of written description.

For these reasons, Applicants request withdrawal of the rejection under 35 U.S.C. §112, ¶1 be withdrawn.

<u>**New Claims**</u>

New claims 51-57 are added herein. The Applicants believe these claims fall within the scope of the elected group. Thus, the Applicants request that the new claims be considered. Support for new claims 51, 52, 53, and 54 can be found, for example, on page 16, lines 6-20 of the present application. Chemical modifications are described at page 16, lines 6-20 of the application. PEGylation and techniques for PEGylation are described in Lundblad and Noyes (1988) *Chemical Reagents for Protein Modifications* as cited at page 16, line 10-12 of the application. Lundblad and Noyes is incorporated by reference at page 55, lines 18-20 of the specification.

Support for new claims 55, 56, and 57 can be found in the specification, for example, on page 8, lines 8-29 (identification of helices); on page 11, lines 21-22 ("Particularly interesting peptides have ends corresponding to structural domain boundaries, e.g., helices A, B, C, and/or D."); and page 33, lines 10-12 (methods using fragments).

These new claims are believed to allowable and allowance is requested.

<u>Conclusion</u>

For the reasons set forth above, the Applicants submit that the claims of this application are allowable. Reconsideration and withdrawal of the Examiner's rejections are hereby requested. Allowance of the claims remaining in this application is earnestly solicited.

In the event that a telephone conversation could expedite the prosecution of this application, the Examiner is requested to call the undersigned at 404-892-5005.

A petition and fee for a one-month extension of time are provided with this Amendment. No additional fees are believed to be due, however, please apply any charges or credits to deposit account 06-1050, referencing Attorney Docket No. 16622-007002.

Respectfully submitted,

Date: May 30, 2007

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Immunology

Biochemical signal transmitted by Fcγ receptors: Phospholipase A_2 activity of Fcγ2b receptor of murine macrophage cell line P388D$_1$

(arachidonic acid/prostaglandin/immunoregulation)

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Communicated by Leon O. Jacobson, September 23, 1981

ABSTRACT The detergent lysate of the P388D$_1$ macrophage cell line was subjected to affinity chromatography on two different media, Sepharose coupled to heat-aggregated human IgG (IgG-Sepharose) and Sepharose coupled to the phosphatidylcholine analog *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine (PC-Sepharose). Both IgG- and phosphatidylcholine-binding proteins were further purified by Sephadex G-100 gel filtration and isoelectric focusing in the presence of 6 M urea. The isolated IgG-binding proteins specifically bound to IgG2a, but not to IgG2b, whereas the isolated phosphatidylcholine-binding proteins specifically bound to IgG2b but not to IgG2a. Phosphatidylcholine-binding proteins possessed a typical phospholipase A_2 activity (phosphatide 2-acylhydrolase, EC 3.1.1.4), which was maximal (16 μmol/min per mg of protein) at pH 8.5, depended on Ca^{2+}, and was specific for cleavage of fatty acid from the C-2 position of the glycerol backbone of phosphatidylcholine. The noted enzymatic activity was augmented 4-fold by preincubating phosphatidylcholine-binding proteins with heat-aggregated murine IgG2b but not with IgG2a. IgG-binding proteins, on the other hand, are devoid of any detectable phospholipase A_2 activity. Thus, the functional significance of Fcγ2b receptor of P388D$_1$ macrophage cell line would be the generation of phospholipase A_2 activity at the cell surface upon specific binding to Fcγ2b fragment.

Fcγ receptor (FcγR) is an integral membrane phospholipoprotein that specifically binds the Fc portion of IgG proteins at the surface of various cells, including B lymphocytes and macrophages (1–7). The precise function of FcγR at the cell surface in the immune response is not yet clearly defined, with one notable exception. This is FcγRs present on K cells, which were shown to be directly involved in antibody-dependent cell-mediated cytotoxicity (8, 9).

A cell surface receptor is defined as a molecule that transmits, upon specific binding of ligand, a signal that affects cell functions. Immune complexes have been known to suppress humoral immune responses (10) or B cell differentiation (11). Such suppression could result from the increased synthesis of prostaglandins (PGs) of the E series, which are potent inhibitors of cell function (12–14), because a marked increase of PGE$_2$ synthesis by human as well as murine peritoneal macrophages upon interaction of cell surface FcγR with Fcγ fragment or immune complexes has been reported (15–17). One of the initial rate-limiting steps of PG synthesis is the activation of phospholipase A_2 (EC 3.1.1.4), which catalyzes hydrolysis of the ester bond at the C-2 position of phospholipids to release an unsaturated fatty acid such as arachidonic, the precursor of PGs (18). If the specific binding of the Fcγ portion to FcγR at the cell surface activates phospholipase A_2, FcγR has to be either closely associated with or even identical to phospholipase A_2. Our previous studies (19) have shown that FcγR proteins isolated from human B cells are indeed bifunctional—i.e., endowed with both Fc-binding and phospholipase A_2 activity.

In this study, the question of whether or not FcγR proteins present on macrophages of different species also possess phospholipase A_2 activity is raised. The data in this paper will demonstrate that the proteins isolated from the detergent lysate of P388D$_1$ murine macrophage cell line by affinity chromatography on PC-Sepharose 4B [which is *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine, a phosphatidylcholine (PtdCho) analog, coupled to Sepharose 4B] bind specifically to murine IgG2b and also possess phospholipase A_2 activity, which is augmented by the binding of heat-aggregated IgG2b. The materials isolated by affinity chromatography on IgG-Sepharose 4B bind specifically to murine IgG2a and are devoid of phospholipase A_2 activity.

MATERIALS AND METHODS

Cells. Murine macrophage cell line (P388D$_1$) was a gift of H. Koren of Duke University. Cells were cultured in a spinner flask at 37°C in an atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with fetal calf serum (10%), streptomycin (100 mg/ml) and penicillin (100 units/ml). Cell density was maintained at approximately 5×10^5 per ml. About 90% of these cells were FcγR$^+$, as determined by the rosette assay using the system of human or sheep erythrocytes coated with IgG antibody (EAγ system). Normal human peripheral blood mononuclear cells were obtained from heparin-treated blood by Ficoll/Hypaque centrifugation (20). Hybridoma cell lines (N-S 8.1 and S-S.1, which secrete anti-sheep erythrocyte antibody of IgG2b and IgG2a subclasses, respectively) were obtained from the Cell Distribution Center of the Salk Institute (San Diego, CA) and cultured in the medium described above.

Surface Radioiodination and Detergent Lysis of Cells. Cultured cells were radioiodinated with 2 mCi (1 Ci = 3.7×10^{10} becquerels) of ^{125}I (Amersham) by the lactoperoxidase-catalyzed method (21), using Enzymobeads (Bio-Rad) (22) as described (19). Cells including 40–50% radioiodinated cells were suspended in phosphate-buffered saline (P_i/NaCl) containing 25 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PhMeSO$_2$F) at 0°C and were lysed with 1% Triton X-100 (Amersham). After stirring for 30 min, nuclear materials, unlysed cells, and debris were removed by centrifugation at 22,370 × g for 60 min at 5°C. The supernatant solution, designated as cell lysate, was immediately subjected to affinity chromatography.

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Abbreviations: EAγ, IgG class antibody-coated erythrocytes; FcγR, Fcγ receptor; IgG-Sepharose, heat-aggregated human IgG-Sepharose 4B conjugate; PC-Sepharose 4B, *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine coupled to Sepharose 4B; PG, prostaglandin; PhMeSO$_2$F, phenylmethylsulfonyl fluoride; P_i/NaCl, phosphate-buffered saline; PtdCho, phosphatidylcholine; sRBC, sheep erythrocytes; Tris/NaCl, Tris·HCl-buffered saline.

Affinity Chromatography. PC-Sepharose 4B, the medium used to extract phospholipase A_2, was prepared as described (19). Normal human IgG proteins, Fabγ and Fcγ fragments, heat-aggretated IgG, and heat-aggregated IgG-Sepharose 4B conjugate were prepared as described (6, 7). Staphylococcal protein A-Sepharose CL-4B conjugate was purchased from Pharmacia (Uppsala, Sweden). All affinity chromatography media were packed in glass columns and equilibrated with Tris·HCl-buffered saline (Tris/NaCl) containing Triton X-100 (0.5%) and $PhMeSO_2F$ (1 mM). After thorough washing with the same buffer, the bound materials were eluted with deionized 6 M urea made 0.2 in ionic strength, pH 8 Tris·HCl buffer containing $PhMeSO_2F$ (1 mM).

Other Physicochemical Methods. The method of isoelectric focusing in the presence of 6 M urea has been described (6, 7, 19). Approximate estimations of protein concentrations in Triton X-100-containing buffer was made by Coomassie blue colorimetry (23) using a Bio-Rad protein assay kit. The more precise determination of protein concentration was carried out by nitrogen analysis (Kjeldahl), assuming the nitrogen contents of the protein samples to be 16%. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ followed the method of Weber and Osborne (24).

Assay of Phospholipase A_2 Activity. The rate of hydrolysis of PtdCho (Sigma) by various preparations in the assay solution (5 mM $CaCl_2$/20 mM KCl/0.5% Triton X-100) was followed by titration with 5 mM NaOH in a pH-stat titrator (19).

The positional specificity of phospholipase A_2 activity was assessed by measuring the levels of the radioactive oleic acid cleaved from 2-[^{3}H]oleoyl PtdCho by FcγR materials in the presence or absence of various IgG preparations as described (19). Preparation of 2-[^{3}H]oleoyl PtdCho followed the method of Robertson and Lands (25).

EAγ Rosette Assay. Human EAγ was prepared as described (6, 7, 19). Murine EAγ was prepared by sensitizing sheep erythrocytes (sRBC) with subagglutinating dose of murine monoclonal anti-sRBC antibodies (IgG2a or IgG2b). EAγ rosetting with human peripheral mononuclear cells or $P388D_1$ cells suspended in P_i/NaCl (1×10^6 cells per ml) and inhibition of EAγ rosetting systems with various IgG preparations were examined as described (6, 7, 19). Individual assays performed in triplicate had a standard error of less than 10%.

RESULTS

Isolation of IgG- and PtdCho-Binding Proteins from $P388D_1$ Cell Lysate. The presence of separate FcγR for IgG2a and IgG2b on the plasma membrane of murine macrophages and of macrophage cell lines has been suggested by several laboratories (26–32). If these FcγRs possess phospholipase A_2 activity as do human B cell FcγRs (19), they should bind specifically not only to IgG, but also to PC-Sepharose, an affinity chromatography medium made with a specific substrate analog.

In the first experiment, the lysate of 3×10^9 cells (40% of cells surface radioiodinated) was first adsorbed on IgG-Sepharose. The unbound material was then passed through a PC-Sepharose column in the presence of 25 mM Ca^{2+}. On the basis of the trichloroacetic acid-precipitable radioactivity, about 0.4% and 1.3% of the original material were obtained as IgG- and PtdCho-binding proteins, respectively. In the second experiment, the order of the affinity chromatography was reversed with the lysate of the same number of cells (53% of cells surface radioiodinated). The yields of the materials that could be eluted from PC- and IgG-Sepharose were equivalent to the first experiment. The IgG- and the PtdCho-binding proteins obtained from the above two experiments were pooled separately and were subjected to gel filtration using a column (5 × 60 cm) of Sephadex G-100 that was previously equilibrated with the deionized 6 M urea made 0.2 in ionic strength, pH 8 Tris·HCl buffer with 1 mM $PhMeSO_2F$. About 90% of PtdCho-binding proteins applied to this column were eluted in the void volume. About 85% of IgG-binding proteins were similarly excluded from Sephadex G-100 gel beads.

PtdCho- and IgG-binding proteins obtained as the excluded fractions from Sephadex G-100 gel were separately dialyzed against deionized water and electrofocused in a pH gradient formed with carrier ampholyte pH 5–10 in the presence of 6 M urea. As illustrated by Fig. 1A, the PtdCho-binding proteins

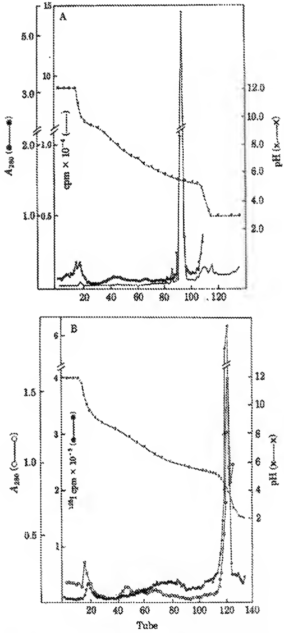

FIG. 1. Isoelectric focusing patterns of the PtdCho-binding proteins originated from the lysate of 6×10^9 $P388D_1$ cells (*A*) and of the IgG-binding proteins originated from the lysate of 1.2×10^9 cells (*B*). Electrofocusing was carried out in an LKB Ampholine column (440 ml) at 1200 V for 72 hr at 10°C in the pH gradient 5–10 in the presence of 6 M urea. Each tube contained 120 drops.

focused sharply at pH 5.8 as a single peak (in tubes 87–94), indicating their charge homogeneity. The IgG-binding proteins were apparently more acidic, because they focused (in tubes 112–122) at approximately pH 4.5, which is in the interphase region between the lower range of the pH gradient and the anode solution (Fig. 1B). IgG-binding proteins used in the subsequent experiments were not electrofocused to avoid possible damage at this low pH. The PtdCho-binding proteins separated by electrofocusing and the IgG-binding proteins fractionated by gel filtration were each extensively dialyzed against deionized water, and then lyophilized. On the basis of the dry weight, the yields of PtdCho- and IgG-binding proteins were approximately 7 and 5 mg per 10^9 cells, respectively.

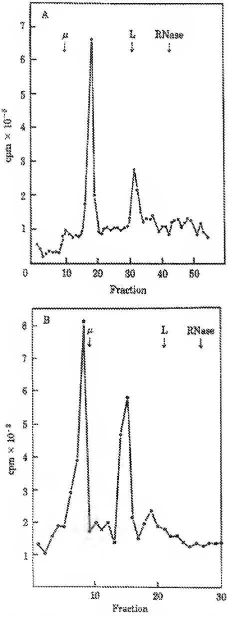

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis patterns of IgG-binding (A) and PtdCho-binding (B) proteins. Electrophoreses were carried out in 7.5% polyacrylamide gels for 6.5 hr (A) and 4 hr (B) at about 8 mA/gel. Fractions are 2-mm gel slices. Positions of IgG μ chain, light chain (L), and RNase markers are shown.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis Patterns. Both IgG- and PtdCho-binding proteins form polymeric complexes in the presence of 6 M urea, as was concluded from the observation that they were excluded from Sephadex G-100 gel beads. On $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing condition, IgG-binding proteins were separated into a major and a minor band, corresponding to molecular weights of 50,000 and 25,000 (Fig. 2A), whereas PtdCho-binding proteins gave rise to two major bands (corresponding to molecular weights of 40,000 and 80,000) (Fig. 2B). Size heterogeneity revealed by $NaDodSO_4$/polyacrylamide gel electrophoresis of both types of FcγR materials isolated from $P388D_1$ cells has been noted by a number of investigators (31–35). The ability of both IgG- and PtdCho-binding proteins to aggregate is probably an intrinsic property of membrane glycoprotein and may be due to strong noncovalent association between hydrophobic segments of their polypeptide chains (36). The association of lipids (7) and carbohydrates (35) with FcγR protein moiety may also contribute to size heterogeneity as a result of anomalous binding of $NaDodSO_4$ (24).

IgG-Binding Properties. The lyophilized IgG- and PtdCho-binding proteins were suspended in P_i/NaCl and examined for their capability to inhibit the murine EAγ rosetting system. As shown by Fig. 3, the IgG-binding proteins inhibited in a dose-dependent manner the rosette formation only between $P388D_1$ cells and EAγ2a systems. On the other hand, the EAγ2b rosette formation was inhibited in a dose-dependent manner by the PtdCho-binding proteins. In addition, as shown by Fig. 4, the EAγ2a rosette inhibition by IgG-binding proteins could be reversed in a dose-dependent manner by preincubating IgG-binding proteins with monomeric IgG2a but not with monomeric IgG2a. The EAγ2b rosette inhibition by PtdCho-binding proteins could be reversed in a dose-dependent manner by preincubating PtdCho-binding proteins with heat-aggregated IgG2b but not with aggregated IgG2a (Fig. 4).

Thus, the isolated IgG- and PtdCho-binding proteins appear to represent the IgG2a and the IgG2b receptors, respectively. Furthermore, at 25 μg/ml both proteins could inhibit 90% of the EAγ rosette formation between human mononuclear cells and human anti-Rh antibody-coated erythrocytes, confirming the lack of species specificity noted previously (33). The inhi-

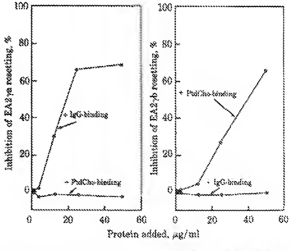

FIG. 3. Inhibition of EAγ rosette formation by the IgG-binding (●) and PtdCho-binding (○) proteins. EAγ was prepared by sensitizing sRBC with the subagglutinating doses of monoclonal anti-sRBC antibodies of IgG2a or IgG2b subclasses. Rosetting cells were $P388D_1$ cells.

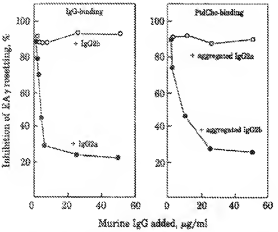

FIG. 4. Reversal of the EAγ rosette inhibitory capacity of the IgG-binding (*Left*) and PtdCho-binding (*Right*) materials by murine IgG preparations. Various amounts of IgG2a or IgG2b (monomeric and heat-aggregated) were preincubated with IgG- or PtdCho-binding proteins (25 μg/ml) before they were added to a EAγ rosette system consisting of monoclonal anti-sRBC-coated sRBC and P388D$_1$ cells. Aggregated IgG2a or IgG2b did not reverse the inhibition by IgG-binding protein. Likewise, monomeric IgG2a and IgG2b failed to reverse the inhibition by PtdCho-binding protein. Data relative to these effects are omitted from this figure for simplification.

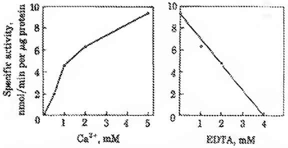

FIG. 5. Activation by Ca^{2+} (*Left*) and inhibition by EDTA (*Right*) of phospholipase A$_2$ activity of the PtdCho-binding proteins.

bition of the human EAγ rosetting system by IgG-binding proteins was reversed again only by the preincubation with monomeric murine IgG2a. Likewise, the inhibitory capacity of PtdCho-binding proteins was abolished only by the preincubation with heat-aggregated IgG2b proteins.

Phospholipase A$_2$ Activity. In order to determine whether or not PtdCho- and IgG-binding proteins possess phospholipase A$_2$ activity, they were first extensively dialyzed against 0.15 M KCl containing 25 mM CaCl$_2$ and 0.5% Triton X-100. By using a pH-stat assay (19), it was found that PtdCho-binding proteins were capable of catalyzing the hydrolysis of PtdCho between pH 7.5 and 10, the optimal pH being near 9.5. IgG-binding proteins, however, showed no activity over the pH 4–10 range. As shown in Fig. 5, the maximal enzymatic activity of PtdCho-binding proteins in the presence of 5 mM Ca^{2+} was about 10 μmol/min per mg. Higher levels of Ca^{2+} resulted in a gradual diminution of enzymatic activity. EDTA at 4 mM totally suppressed the noted enzymatic activity. Mg^{2+} or other divalent cations could not substitute for Ca^{2+}.

Positional Specificity of Phospholipase Activity of the PtdCho-Binding Proteins. The results summarized in Table 1 demonstrate that the PtdCho-binding proteins were capable of hydrolyzing specifically the ester bond at the C-2 position of the glycerol backbone of this substrate, releasing about 20% of the radioactivity as free oleic acid. Preincubation of the PtdCho-binding proteins with heat-aggregated murine IgG2b resulted in the augmentation of the phospholipase A$_2$ activity, with liberation of about 88% of the radioactivity as oleic acid. This level of enzymatic activity was found to be equivalent to that obtained when bee venom phospholipase A$_2$ was used as control. On the other hand, neither IgG2b (monomeric) nor IgG2a (monomeric or heat-aggregated) activated this enzymatic activity. A small amount of the radioactivity found in lyso-PtdCho fractions may have arisen from 1-[^{3}H]oleoyl PtdCho, which is usually present in small quantity (about 1%) in 2-[^{3}H]oleoyl PtdCho.

Table 1. Positional specificity of phospholipase activity of the PtdCho-binding proteins in the presence and absence of two different subclasses (2a and 2b) of murine IgG*

			cpm × 10^{-4} (%)† found in		
Exp.	Enzymes	IgG added	Free fatty acid	PtdCho	Lyso-PtdCho
1	Bee venom phospholipase A$_2$	—	162.0 (93.9%)	10.0 (5.8%)	0.6 (0.3%)
2	PtdCho-binding protein	—	34.2 (20.0%)	136.0 (79.5%)	0.9 (0.5%)
3	PtdCho-binding protein	Aggregated 2b	150.0 (88.2%)	19.0 (11.2%)	1.0 (0.6%)
4	PtdCho-binding protein	Monomeric 2b	33.8 (19.9%)	135.0 (79.6%)	0.7 (0.4%)
5	PtdCho-binding protein	Aggregated 2a	32.0 (19.0%)	136.0 (80.7%)	0.5 (0.3%)
6	PtdCho-binding protein	Monomeric 2a	35.0 (21.0%)	131.5 (78.8%)	0.3 (0.2%)
7	—	Aggregated 2b	0.5 (0.3%)	162.0 (99.5%)	0.3 (0.2%)
8	—	Monomeric 2b	0.5 (0.3%)	167.0 (99.3%)	0.6 (0.4%)
9	—	Aggregated 2a	0.4 (0.2%)	162.0 (99.7%)	0.1 (0.1%)
10	—	Monomeric 2a	0.7 (0.4%)	168.0 (99.6%)	0.08 (0.05%)
11	—	—	0.4 (0.2%)	168.0 (99.6%)	0.17 (0.1%)

* In the experiments numbered 2–6, 40 μg of PtdCho-binding proteins and 120 μg of various murine IgG proteins were preincubated in 250 μl of the assay solution (20 mM KCl containing 5 mM CaCl$_2$ and 0.5% Triton X-100) at 37°C for 30 min. These were added to substrate (10 mg of PtdCho containing a tracer amount of 2-[^{3}H]oleoyl PtdCho suspended in 6 ml of assay solution) in a thermoregulated vessel. The hydrolysis was followed by a pH-stat titrator at pH 9.5 at 37°C for 2 hr. After this, lipids were extracted from the reaction mixtures with chloroform/methanol (2:1, vol/vol). Lipid extracts were fractionated by silicic acid column chromatography as described (19).

† Percentages given are based on the total radioactivity recovered. The recovery of the radioactivity ranged between 85% and 95%.

DISCUSSION

The data presented in this paper demonstrate the separation of two types of FcγR in a biologically active form from the detergent lysate of P388D$_1$ cells. The data of Fig. 3 and 4, which clearly support the subclass specificity in their IgG-binding properties, suggest that the IgG- and the PtdCho-binding proteins isolated represent an Fcγ2aR and an Fcγ2bR, respectively. The charge properties of these proteins are quite different, as illustrated by the data of Fig. 1. This may reflect differences in their carbohydrate contents as well as in amino acid compositions. Preliminary results of tryptic peptide mapping also suggested marked differences between IgG- and PtdCho-binding proteins. Further delineation of the two distinct FcγRs of murine macrophages will ultimately depend on thorough biochemical characterization such as amino acid sequence analysis.

The result of human EAγ rosette inhibition indicated that both Fcγ2aR and Fcγ2bR are able to bind to the Fcγ portion of human IgG. The reason why PtdCho-binding proteins (Fcγ2bR) failed to bind IgG-Sepharose is not clear. A possibility is that Fcγ2bR has much lower affinity for human IgG than does Fcγ2aR. Loube *et al.* (31) reported the isolation of Fcγ2aR but not Fcγ2bR from P388D$_1$ cell lysate by affinity chromatography using human IgG-Sepharose.

The data in this study also demonstrate that phospholipase A_2 activity is an inherent property of PtdCho-binding but not of IgG-binding protein isolated from the P388D$_1$ cell lysates. The enzymatic activity exhibited by PtdCho-binding protein was essentially identical to that of human B cell FcγR protein (19) in pH optimum (pH 9.5), Ca^{2+} dependency (Fig. 5), specific activity (about 10 μmol of fatty acid released per min per mg of protein) and positional specificity (Table 1). However, the definite positional specificity needs to be confirmed by selectively assaying for phospholipase A_1 activity in these materials. The noted enzymatic activity was shown to be augmented about 4-fold by heat-aggregated murine IgG2b proteins but not by monomeric IgG2b or IgG2a (Table 1). These findings strongly suggest that Fcγ2bRs but not Fcγ2aRs are the surface molecules that, upon specific binding of the immune complexes, transmit a signal for increased synthesis of PGE; this synthesis has been shown by Rouzer *et al.* (17) to be independent of endocytosis or phagocytosis. Indeed, results of our preliminary experiments have demonstrated that P388D$_1$ cells radiolabeled with [^{3}H]arachidonic acid release [^{3}H]arachidonic acids and convert them into PGs after the interaction with EAγ2b complexes but not with EAγ2a complexes. An interesting question then presents itself as to the biological significance of Fcγ2aRs which lack any demonstrable phospholipase A_2 activity and yet are able to bind to IgG2a proteins.

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WSX-1 Is Required for the Initiation of Th1 Responses and Resistance to *L. major* Infection

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Summary

WSX-1 is a class I cytokine receptor with homology to the IL-12 receptors. The physiological role of WSX-1, which is expressed mainly in T cells, was investigated in gene-targeted WSX-1-deficient mice. IFN-γ production was reduced in isolated *WSX-1*$^{-/-}$ T cells subjected to primary stimulation in vitro to induce Th1 differentiation but was normal in fully differentiated and activated *WSX-1*$^{-/-}$ Th1 cells that had received secondary stimulation. *WSX-1*$^{-/-}$ mice were remarkably susceptible to *Leishmania major* infection, showing impaired IFN-γ production early in the infection. However, IFN-γ production during the later phases of the infection was not impaired in the knockout. *WSX-1*$^{-/-}$ mice also showed poorly differentiated granulomas with dispersed accumulations of mononuclear cells when infected with bacillus Calmette-Guerin (BCG). Thus, WSX-1 is essential for the initial mounting of Th1 responses but dispensable for their maintenance.

Introduction

When CD4$^+$ Th cells are activated by an encounter with a pathogen, they proliferate and differentiate into either Th1 or Th2 cells, functionally distinct subsets that produce characteristic cytokine profiles (Mosmann and Sad, 1996). Th1 cytokines, especially IFN-γ and TNF-α, are critical for the macrophage activation and nitric oxide production required for eliminating intracellular pathogens such as *Leishmania major* (Nacy et al., 1991; Swihart et al., 1995). In contrast, Th2 cytokines such as IL-4, IL-5, and IL-13 are important for inducing the humoral immunity required to counter helminth infections (Finkelman et al., 1991). Th1 cells and Th2 cells develop from the same Th precursor (Thp) cells, but which subset differentiates in a given situation is driven by factors, particularly cytokines, in the surrounding microenvironment. In vitro, IL-12 promotes IFN-γ production and Th1 development via activation of the signal transducer and activator of transcription 4 (STAT4), whereas IL-4 binding to the IL-4 receptor (IL-4R) promotes IL-4 production and Th2 development via activation of STAT6 (Murphy et al., 2000).

Receptors for most interleukins and cytokines are multichain complexes with high affinity for specific cytokines. Many receptor complex components belong to the class I cytokine receptor family (Bazan, 1990). These proteins contain at least two fibronectin type III-like domains: an N-terminal domain with four conserved cysteine residues and a second domain featuring a Trp-Ser-X-Trp-Ser (WSXWS) motif (Miyajima et al., 1992). The high-affinity receptor complex often contains a cytokine-specific receptor protein and a common signal-transducing component, both of which may belong to the class I cytokine receptor family. The signaling protein may be shared between several receptor complexes. For example, gp130 was originally described as the signaling component of IL-6R, but it is also found in receptors for IL-6-related cytokines such as IL-11, leukemia inhibitory factor, and cardiotropin (Taga et al., 1989). Similarly, the common β chain is shared by the receptors for IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (Miyajima, 1992), whereas the common γ chain is shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (Di Santo et al., 1995). By engaging receptor complexes in which one of the common subunits is combined with a cytokine-specific chain, cytokines exert multifunctional, pleiotropic, and sometimes redundant roles in hematopoietic and lymphoid systems (Paul, 1989).

WSX-1 (Sprecher et al., 1998) is a novel class I cytokine receptor containing Trp-Gly-Glu-Trp-Ser (WGEWS), a sequence that fits the WSXWS motif (Miyazaki et al., 1991). WSX-1 is highly expressed in spleen, thymus, and lymph nodes, particularly in the CD4$^+$ T cell compartment. WSX-1 was first cloned from an EST database using the human gp130 sequence as a query. WSX-1 is identical to the TCCR molecule cloned by Chen et al. (2000). At the amino acid level, WSX-1/TCCR is 19% identical to the gp130 protein but, strikingly, is 26% identical to the IL-12Rβ2 chain (Sprecher et al., 1998; Chen et al., 2000).

To define the role of WSX-1/TCCR in vivo, we generated *WSX-1/TCCR* knockout mice using homologous recombination. Although development of the hematopoietic and lymphoid systems in *WSX-1*$^{-/-}$ mice was normal, isolated T cells from the mutant animals produced reduced levels of IFN-γ when treated in vitro with IL-12 plus Concanavalin A (ConA) or anti-CD3 antibody,

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However, to our surprise, fully differentiated *WSX-1*$^{-/-}$ Th1 cells subjected to a secondary stimulation with ConA produced wild-type levels of IFN-γ. Moreover, although *WSX-1*$^{-/-}$ mice exhibited reduced IFN-γ production in the early stages of *L. major* infection and were remarkably susceptible to the pathogen, there was no impairment of IFN-γ production during the later phases. The knockout mice also showed impaired granuloma formation when infected with BCG but showed normal serum IFN-γ production later in the infection. Our results indicate that WSX-1/TCCR is critical for the initial production of IFN-γ following infection with an intracellular pathogen but is not required for the maintenance of the Th1 response.

Results

Generation of *WSX-1*$^{-/-}$ Mutant Mice

The *WSX-1* gene was disrupted in murine embryonic stem (ES) cells using a targeting vector in which an exon encoding a part of the second fibronectin type III domain was deleted (see Experimental Procedures and Figure 1A). Mice heterozygous for the *WSX-1* mutation were generated from ES cells and chimeric C57BL/6 mice using standard procedures. Heterozygous *WSX-1*$^{+/-}$ mice were healthy and fertile, and homozygous *WSX-1*$^{-/-}$ mice were born to heterozygous intercrosses at the expected Mendelian ratio (Figures 1B and 1C). Two independent strains of *WSX-1*$^{-/-}$ mice, derived from different heterozygous ES cell clones, showed similar phenotypes. *WSX-1*$^{-/-}$ mice were healthy and fertile, and there were no significant differences in gross or radiographic findings or in body or organ weights among wild-type, heterozygous, and homozygous mice (data not shown). Reverse transcription-PCR (RT-PCR) analysis showed that *WSX-1* was expressed strongly in wild-type CD4^{+} T cells, weakly in CD8^{+} T cells and B220^{+} B cells, and minimally in plastic-adherent splenic macrophages (Figure 1D), which is in line with previous reports (Sprecher et al., 1998; Chen et al., 2000). The null mutation of *WSX-1* in *WSX-1*$^{-/-}$ mice was confirmed by the absence of WSX-1 protein in Western blots of splenic cell lysates (Figure 1E).

Normal Hematopoietic and Lymphoid Development in *WSX-1*$^{-/-}$ Mice

WSX-1 is highly similar to gp130, a cytokine receptor signaling component that is critical for hematopoiesis (Taga and Kishimoto, 1997). This fact, plus the expression pattern of WSX-1 in lymphoid cells and bone marrow, led us to investigate the development of hematopoietic cells in *WSX-1*$^{-/-}$ mice. As summarized in Table 1, no significant differences in the numbers of WBC, RBC, or platelets or in hemoglobin concentration were observed among *WSX-1*$^{+/+}$, *WSX-1*$^{+/-}$, and *WSX-1*$^{-/-}$ mice. Differential counts of WBC revealed that the percentages and numbers of neutrophils, lymphocytes, monocytes, and eosinophils were comparable among the three groups of mice (data not shown). Furthermore, there were no significant differences in serum chemistry values such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels (data not shown).

We then examined the development of lymphoid or-

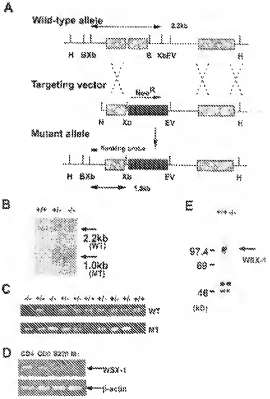

Figure 1. Generation of *WSX-1*$^{-/-}$ Mice

(A) A portion of the mouse *WSX-1* wild-type locus is shown with exons (hatched boxes) and a 2.2 kb XbaI fragment diagnostic for the wild-type allele (top). The targeting vector was designed to replace an exon encoding a portion of the extracellular fibronectin type III domain with a neomycin resistance cassette (closed box) (middle). The mutated WSX-1 locus contains a 1.0 kb XbaI fragment. The position of the 5′ flanking probe used for Southern blot analysis is shown (bottom). H, B, Xb, and EV represent HindIII, Bam HI, XbaI, and EcoRV sites, respectively.

(B) Southern blot analysis. Genomic DNA from wild-type mice (+/+) or mice heterozygous (+/−) or homozygous (−/−) for the *WSX-1* mutation was digested with Xba I and hybridized to the 5′ flanking probe. The 2.2 kb wild-type fragment (WT) and the 1.0 kb mutant fragment (MT) are indicated.

(C) A representative PCR genotyping of a litter born to a *WSX-1* heterozygous intercross is shown. +/+, wild-type; +/−, heterozygote; −/−, homozygote.

(D) An RT-PCR analysis of normal *WSX-1* expression. CD4^{+} T cells, CD8^{+} T cells, B220^{+} B cells, and plastic-adherent splenic macrophages (Mφ) were obtained from wild-type C57BL/6 mice. Expression of *WSX-1* was analyzed using RT-PCR. Expression of β-actin was analyzed as an internal control.

(E) Western blot analysis of WSX-1 expression. Lysates of wild-type (+/+) or knockout (−/−) spleen cells were immunoprecipitated using anti-WSX-1 antiserum.

gans in the absence of WSX-1. There were no significant differences in the numbers of thymocytes, lymph node cells, or spleen cells in *WSX-1*$^{-/-}$ mice compared with controls (data not shown). Furthermore, flow cytometric analysis showed that the development and differentiation of lymphocytes in the thymus, spleen, lymph nodes, and bone marrow were normal (Figure 2 and data not shown). The development of intestinal intraepithelial lymphocytes and Peyer's patches was also normal (data

Table 1. Hematological Examination of *WSX-1*$^{-/-}$ Mice

Genotype	*WSX-1*$^{+/+}$	*WSX-1*$^{+/-}$	*WSX-1*$^{-/-}$
WBC ($\times 10^3/\mu l$)	3.76 ± 0.85	4.02 ± 0.99	4.93 ± 1.76
RBC ($\times 10^6/\mu l$)	9.83 ± 0.22	9.20 ± 0.95	9.62 ± 0.69
HGB (g/dl)	15.4 ± 0.3	15.3 ± 1.1	15.6 ± 1.0
PLT ($\times 10^3/\mu l$)	1165 ± 81	1214 ± 151	1175 ± 235

The numbers of WBC, RBC, and platelets (PLT) and the concentration of hemoglobin (HGB) were examined in 6- to 8-week-old mice (4 mice per group).

not shown). Because gp130 is crucial for heart development (Yoshida et al., 1996) and WSX-1 is reportedly expressed in the heart (Sprecher et al., 1998), we examined this organ in *WSX-1*$^{-/-}$ mice. The heart was found to be normal in *WSX-1*$^{-/-}$ mice by macroscopic inspection (data not shown). Our data thus demonstrate that an absence of WSX-1 does not affect the development of the heart or the hematopoietic or lymphoid systems.

Hyperproliferation of *WSX-1*$^{-/-}$ T Cells

Because WSX-1 is preferentially expressed in T cells, the proliferation in vitro of T cells from *WSX-1*$^{-/-}$ mice was evaluated. The proliferation of *WSX-1*$^{-/-}$ splenocytes was increased slightly over that of wild-type splenocytes when the cells were stimulated with either increasing concentrations of anti-CD3 antibody (Figure 3A) or ConA (data not shown). In agreement with this finding, T cells from *WSX-1*$^{-/-}$ mice showed a relative increase in the number of cells in the S and G2+M phases of the cell cycle compared with wild-type when the cells were stimulated with anti-CD3 plus anti-CD28 antibodies (47.3% and 9.5% in the mutant, respectively, versus 40.8% and 8.9% in the wild-type) (Figure 3B). The high homology of WSX-1 to the IL-12 receptor prompted us to examine the proliferation of *WSX-1*$^{-/-}$ T cells in response to treatment with anti-CD3 antibody plus increasing concentrations of IL-12 (Figure 3C). However, after taking the higher baseline of *WSX-1*$^{-/-}$ T cell proliferation into account, *WSX-1*$^{-/-}$ T cells stimulated with IL-12 showed the same dose-response kinetics as *WSX-1*$^{+/+}$ T cells (Figure 3C, left). When anti-CD28 antibody was added to the treatment, the hyperproliferation of the *WSX-1*$^{-/-}$ T cells was increased over the wild-type at all but the highest concentration of IL-12 (Figure 3C, center and right). These data indicate that WSX-1 may normally have an inhibitory influence on T-cell proliferation and that cells lacking this protein are capable of responding to IL-12.

Impaired IFN-γ Production In Vitro by Differentiating *WSX-1*$^{-/-}$ Th1 Cells

Signals mediated through IL-12R are pivotal for the differentiation of Thp cells into the Th1 type and IFN-γ production. Because of the homology of WSX-1 to the IL-12Rβ2 chain, we examined the role of WSX-1 in the production of IFN-γ as well as in the development of Th1 and Th2 cells using an in vitro differentiation system. Purified CD4$^+$ T cells were treated for 3 days with either ConA, anti-IL-4 antibody and IL-2 plus titrated doses of IL-12 to induce Th1 cell development, or ConA and IL-2 plus IL-4 to induce Th2 cell development (primary stimu-

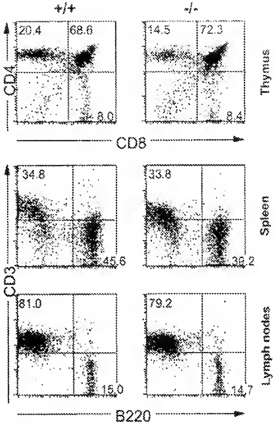

Figure 2. Flow Cytometric Analysis of Thymus, Spleen, and Lymph Node Cells

Single-cell suspensions of thymus, spleen, or lymph node cells from wild-type (+/+) or *WSX-1*$^{-/-}$ (−/−) mice were stained with anti-CD4 versus anti-CD8 or anti-B220 versus anti-CD3, and surface expression was analyzed by flow cytometry. Percentages of positive cells within a quadrant are indicated. Experiments were repeated three times with similar results.

lation). These cells were then restimulated with ConA (secondary stimulation) to induce Th1 or Th2 cytokine production. The supernatants from both the primary and secondary stimulation cultures were analyzed for cytokine profiles. *WSX-1*$^{-/-}$ CD4$^+$ T cells in the Th1 primary culture ("primary cells") produced approximately 3-fold less IFN-γ than similarly treated wild-type CD4$^+$ T cells (Figure 3D). Production of IFN-γ by "primary" *WSX-1*$^{-/-}$ Th1 cells was also 2-fold less than that of wild-type T cells when stimulated with anti-CD3 antibody at 10 μg/ml (data not shown). In striking contrast, the production of IFN-γ by *WSX-1*$^{-/-}$ CD4$^+$ Th1 cells in the secondary stimulation culture was just as vigorous as that of *WSX-1*$^{+/+}$ controls (Figure 3E). Both *WSX-1*$^{-/-}$ and wild-type "secondary" Th1 cells also produced equivalent amounts of IFN-γ in response to the addition of IL-12 (3.5 ng/ml) and/or IL-18 (10 ng/ml) (data not shown). Furthermore, wild-type and *WSX-1*$^{-/-}$ CD4$^+$ T cells cultured in the presence of 3.5 ng/ml IL-12 for 7 days produced equivalent amounts of IFN-γ (WT, 8469.2 ± 822.1 pg/ml; *WSX-1*$^{-/-}$, 9715.3 ± 702.6 pg/ml). Production of IL-4 by *WSX-1*$^{-/-}$ and *WSX-1*$^{+/+}$ Th1 cells was equally suppressed when the cells were cultured with high con-

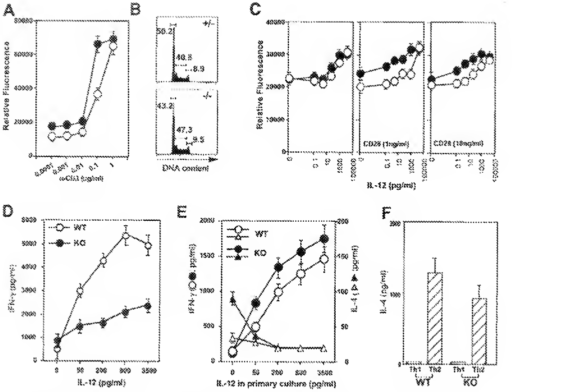

Figure 3. Responses of $WSX\text{-}1^{-/-}$ T Cells to TCR Stimulation and Cytokine Production during Th1 or Th2 Differentiation

(A) Proliferation in response to anti-CD3 stimulation. Spleen cells from wild-type (open circles) or $WSX\text{-}1^{-/-}$ (closed circles) mice were stimulated with plate-bound anti-CD3 for 72 hr. Proliferation was determined 4 hr later by Alamar blue staining. Data shown are mean ± SD of triplicate cultures and are representative of three independent experiments.

(B) Cell cycle analysis. Purified spleen T cells were stimulated with plate-bound anti-CD3 plus soluble anti-CD28 antibodies for 48 hr. The DNA content of individual nuclei was determined by PI staining. Percentages of cells in G0+G1, S, and G2+M phases are indicated from left to right. +/− = $WSX\text{-}1^{+/-}$; −/− = $WSX\text{-}1^{-/-}$. Data are representative of two independent experiments.

(C) IL-12 responsiveness. Spleen cells from wild-type (open circles) or $WSX\text{-}1^{-/-}$ (closed circles) mice were stimulated with 0.1 μg/ml plate-bound anti-CD3 antibody plus increasing concentrations of IL-12 alone (left panel), or plus 1 ng/ml (middle panel), or 10 ng/ml (right panel) anti-CD28 antibody. Proliferation was determined after 72 hr by Alamar blue staining. Data shown are mean ± SD of triplicate cultures and are representative of two independent experiments.

(D) IFN-γ production by Th1 cells after primary stimulation. $CD4^+$ lymph node T cells from wild-type (open circles) or $WSX\text{-}1^{-/-}$ (closed circles) mice were stimulated with the indicated concentrations of IL-12 plus ConA (2.5 μg/ml), IL-2 (50 U/ml), and anti-IL-4 (500 ng/ml) in the presence of irradiated syngeneic spleen cells for 72 hr. Culture supernatants were collected and IFN-γ production determined by ELISA. Data shown are mean ± SD of triplicate cultures and are representative of three independent experiments.

(E) IFN-γ and IL-4 production by Th1 cells after secondary stimulation. $CD4^+$ lymph node T cells from wild-type (open symbols) or $WSX\text{-}1^{-/-}$ (closed symbols) mice were activated as in (D). After 3 days, cells were washed, counted, and restimulated with ConA (2.5 μg/ml) without any additional cytokines for 24 hr. To determine IL-4 production by Th1 cells, cells were subjected to primary and secondary stimulations as above but without anti-IL-4. Culture supernatants were analyzed for the production of IFN-γ (circles) or IL-4 (triangles). X axis values indicate the IL-12 concentrations used for the primary stimulations. Data shown are mean ± SD of triplicate cultures and are representative of three independent experiments.

(F) IL-4 production by Th1 and Th2 cells after secondary stimulation. $CD4^+$ lymph node T cells from wild-type (WT) or $WSX\text{-}1^{-/-}$ (KO) mice were activated in the presence of irradiated syngeneic spleen cells and ConA (2.5 μg/ml). For primary stimulation, the culture medium was supplemented with IL-2 (50 U/ml) plus IL-12 (3.5 ng/ml) for Th1 induction (open columns), or IL-2 (50 U/ml) plus IL-4 (1000 U/ml) for Th2 induction (hatched columns). After 3 days, cells were washed, counted, and restimulated with ConA (2.5 μg/ml) without any additional cytokines for 24 hr. Culture supernatants were collected and analyzed by ELISA for the production of IL-4. Data shown are mean ± SD of triplicate cultures and are representative of four independent experiments.

centrations of IL-12 in the primary culture, but $WSX\text{-}1^{-/-}$ cells produced more IL-4 than wild-type cells when stimulated with no or low concentrations of IL-12 (Figure 3E). The production of IL-4 by in vitro differentiated and restimulated $WSX\text{-}1^{-/-}$ Th2 cells was normal (Figure 3F). Thus, WSX-1 plays a role in inducing Th1 differentiation but is not required for the production of IFN-γ by restimulated Th1 cells.

Susceptibility to *L. major* Infection and Aberrant Cytokine Production in $WSX\text{-}1^{-/-}$ Mice

IFN-γ production is critical for elimination of the intracellular parasite *L. major* (Swihart et al., 1995; Mattner et al., 1996; Park et al., 2000). BALB/c mice are highly susceptible to *L. major* infection because these animals fail to mount the Th1 responses necessary for sustained IFN-γ production. We therefore examined the effects of

WSX-1 deficiency on the course of *L. major* infection. *WSX-1*$^{-/-}$, *WSX-1*$^{+/+}$, and BALB/c mice were subcutaneously infected with *L. major* in the right hind footpad, and footpad swelling was monitored for up to 6 weeks after infection. *WSX-1*$^{-/-}$ mice were clearly more susceptible to *L. major* infection than *WSX-1*$^{+/+}$ mice as indicated by increased footpad swelling (Figure 4A) and the presence of severe ulceration (Figure 4B). Furthermore, the parasite burden in the infected footpad correlated with the degree of footpad swelling (data not shown). However, *WSX-1*$^{-/-}$ mice were not as affected as the susceptible BALB/c mice (Figure 4A). Examination of the progeny within litters born to crosses of *WSX-1*$^{-/-}$ and *WSX-1*$^{+/-}$ mice showed that *WSX-1*$^{-/-}$ mice were more susceptible to *L. major* infection than resistant *WSX-1*$^{+/-}$ littermates (data not shown). These results demonstrate that *WSX-1*$^{-/-}$ mice have a genuine increase in susceptibility to *L. major* infection that is not due to genetic chimerism.

We then examined the Th1 response in vivo by determining IFN-γ production of CD4$^+$ T cells from *L. major*-infected *WSX-1*$^{-/-}$ and *WSX-1*$^{+/-}$ mice. CD4$^+$ T cells were isolated from popliteal lymph nodes (LN) of infected mice 2 weeks after *L. major* infection and stimulated in vitro with *L. major* antigen. IFN-γ production by *WSX-1*$^{-/-}$ T cells was greatly reduced compared with controls at this point (Figure 4C). RT-PCR analyses revealed that the *WSX-1*$^{-/-}$ T cells expressed less mRNA for IFN-γ (Figure 4D) but more mRNA for IL-4 and IL-13 (data not shown) than wild-type cells. These results indicate that Th2 rather than Th1 differentiation occurs in vivo in the absence of WSX-1. Consistent with these findings, the percentage of IFN-γ-producing CD4$^+$ T cells was 2-fold less in *WSX-1*$^{-/-}$ mice than in wild-type mice, as determined by flow cytometric analysis of popliteal LN populations (Figure 4E). To our surprise, however, both the increased susceptibility to infection and the impaired IFN-γ production were evident only in the early phase of the infection. As shown in Figure 4A, footpad swelling had decreased in some *WSX-1*$^{-/-}$ mice by 5–6 weeks postinfection, whereas all BALB/c mice continued to suffer from dramatic footpad swelling and severe ulcerations throughout the infection. Moreover, popliteal LN CD4$^+$ T lymphocytes isolated from *WSX-1*$^{-/-}$ mice 4 weeks or more after infection and stimulated in vitro with *L. major* antigen produced amounts of IFN-γ comparable to those of wild-type cells (Figure 4F). RT-PCR analyses confirmed that CD4$^+$ T lymphocytes isolated from four different *WSX-1*$^{-/-}$ mice and stimulated with *L. major* antigen in vitro expressed normal amounts of IFN-γ mRNA at 4 weeks postinfection (Figure 4G), although the mutant animals themselves showed various degrees of footpad swelling (mouse 1, 1.75 mm; mouse 2, 2.10 mm; mouse 3, 2.20 mm; and mouse 4, 1.80 mm at 4 weeks postinfection). RT-PCR analyses also revealed that *WSX-1*$^{-/-}$ CD4$^+$ T lymphocytes expressed more IL-4 mRNA than wild-type cells at 4 weeks postinfection (Figure 4G).

The kinetics of IFN-γ and IL-4 expression during *L. major* infection were quantitatively examined by competitive PCR (Figure 4H). IFN-γ expression was impaired in *WSX-1*$^{-/-}$ cells in the early phases of the infection (at day 5 and 2 weeks postinfection) but was restored to the wild-type level at 4 and 6 weeks postinfection. In contrast, IL-4 expression by *WSX-1*$^{-/-}$ cells was comparable to the wild-type on day 5 postinfection but elevated compared with the wild-type at 2 weeks postinfection and thereafter. Consistent with this deviation toward a Th2 cytokine profile, *L. major*-infected *WSX-1*$^{-/-}$ mice had elevated serum levels of IgG1 and IgE at 9 weeks postinfection (Figure 4I). These isotypes are highly dependent on Th2 help for class switching. Serum levels of IgG2a, an isotype dependent on IFN-γ production, were normal in *WSX-1*$^{-/-}$ mice at 9 weeks postinfection. We conclude that the initial Th1 response induced by *L. major* infection is impaired in the absence of WSX-1 but that normal levels of IFN-γ can be produced at later time points to protect the animal.

Abnormal Granuloma Formation in *WSX-1*$^{-/-}$ Mice Infected with *Mycobacteria bovis* BCG

Protection against mycobacteria depends on the development of Th1 cells and the production of IFN-γ (Orme et al., 1992). To further examine the effect of WSX-1 deficiency on the elimination of intracellular pathogens, we infected mice with the avirulent strain *M. bovis* BCG. At 2 weeks postinfection, there were approximately eight times more granulomas in the livers of mutant animals compared with controls (Figure 5A). Moreover, the granulomas in *WSX-1*$^{-/-}$ livers were abnormally large and not well differentiated, being poorly demarcated and composed of dispersed accumulations of mononuclear cells (Figure 5B). In contrast, granulomas in *WSX-1*$^{+/+}$ mice were compact with dense accumulations of mononuclear cells. The granuloma phenotype in *WSX-1*$^{-/-}$ mice resembles that observed in *IL-12p40*$^{-/-}$ mice (Cooper et al., 1997). With respect to IFN-γ production, spleen cells from *WSX-1*$^{-/-}$ mice produced less IFN-γ in response to anti-CD3 plus IL-12 than wild-type cells on day 2 postinfection (Figure 5C, left). However, by day 7 postinfection, IFN-γ production by *WSX-1*$^{-/-}$ spleen cells was restored to normal levels. Although serum IFN-γ could not be detected in either *WSX-1*$^{-/-}$ or wild-type mice at 2, 4, or 7 days postinfection, serum levels of IFN-γ were equivalent in wild-type and *WSX-1*$^{-/-}$ mice at 2 weeks postinfection (Figure 5C, right). Despite the poorly differentiated granulomas in the mutant mice, no significant difference in the number of liver CFU was observed between *WSX-1*$^{+/+}$ and *WSX-1*$^{-/-}$ mice (Figure 5D). There was also no significant difference between *WSX-1*$^{-/-}$ and *WSX-1*$^{+/+}$ mice in liver damage as determined by serum AST and ALT levels (Figure 5E). These results show that WSX-1 is required for proper granuloma formation during BCG infection but is not essential for liver protection.

Discussion

In this study, we have demonstrated that the class I cytokine receptor WSX-1/TCCR is critical for normal IFN-γ production by differentiating Th1 cells and resistance to *L. major* but only at the early stages of stimulation or infection. The absence of WSX-1/TCCR in mice neither impairs the completion of in vitro Th1 differentiation, nor abolishes in vivo production of IFN-γ during the later phases of *L. major* or BCG infection. Despite the homology of WSX-1/TCCR to gp130, *WSX-1*$^{-/-}$ mice

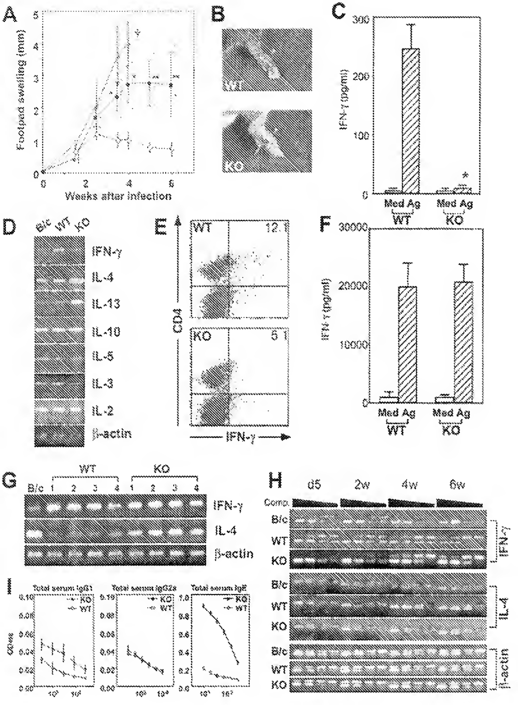

Figure 4. Course of *L. major* Infection in *WSX-1*$^{-/-}$ Mice

(A) Footpad swelling in response to *L. major* infection. BALB/c (open triangles; a susceptible strain), *WSX-1*$^{+/+}$ (open circles), and *WSX-1*$^{-/-}$ mice (closed circles) were inoculated in the right hind footpad with *L. major* promastigotes, and the size of the footpad lesion was monitored as described in Experimental Procedures. Data shown are mean ± SD and are representative of four independent experiments with three to five mice per group. Red symbols are values for individual *WSX-1*$^{-/-}$ mice. †BALB/c mice were sacrificed at 4 weeks for ethical reasons. *$p < 0.001$ and **$p < 0.005$ compared with *WSX-1*$^{+/+}$ mice.

(B) Histology of footpad lesions in *WSX-1*$^{+/+}$ (WT) and *WSX-1*$^{-/-}$ (KO) mice 4 weeks after *L. major* infection. Note the mild swelling in the wild-type footpad but severe swelling (arrowhead) and ulceration (arrow) in the mutant footpad.

(C) IFN-γ production by popliteal LN CD4$^+$ T cells from *WSX-1*$^{+/+}$ (WT) and *WSX-1*$^{-/-}$ (KO) mice 2 weeks after *L. major* infection. CD4$^+$ T cells were cultured with irradiated naive wild-type splenocytes with (hatched columns) or without (open columns) *L. major* antigen (Ag) for 66 hr. IFN-γ concentration in the culture supernatants was measured by ELISA. Data shown are mean ± SD of triplicate samples from four mice

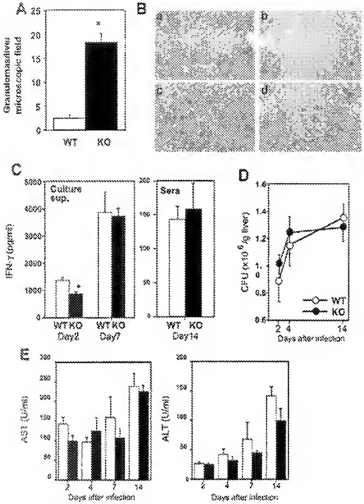

Figure 5. Granuloma Formation in BCG-Infected $WSX\text{-}1^{-/-}$ Mice

(A) $WSX\text{-}1^{+/+}$ (WT) and $WSX\text{-}1^{-/-}$ (KO) mice were infected with BCG. On day 14 after infection, numbers of granulomas in livers of the mice were measured as described in Experimental Procedures. Data shown are the mean ± SD from seven mice per group and are representative of two independent experiments. *$p < 0.001$.

(B) Histology of BCG-infected livers. Fixed thin sections from the $WSX\text{-}1^{+/+}$ (a and c) and $WSX\text{-}1^{-/-}$ (b and d) mice in (A) were stained with hematoxylin and eosin. Magnifications: ×20 (a and b); ×100 (c and d).

(C) IFN-γ production. Spleen cells were prepared from $WSX\text{-}1^{+/+}$ (WT) and $WSX\text{-}1^{-/-}$ (KO) mice on days 2 and 7 postinfection with BCG and cultured with plate-bound anti-CD3 antibody (5 μg/ml) plus IL-12 (1 ng/ml) (left panel). IFN-γ production in culture supernatants was measured by ELISA. IFN-γ concentration in sera from BCG-infected $WSX\text{-}1^{+/+}$ (WT) and $WSX\text{-}1^{-/-}$ (KO) mice measured by ELISA on day 14 postinfection (right panel). No detectable serum IFN-γ was produced in either wild-type or $WSX\text{-}1^{-/-}$ mice on days 2, 4, and 7 postinfection. Data shown are mean ± SD for seven mice per group and are representative of two independent experiments. *$p < 0.05$.

(D) Numbers of CFU in livers of the mice in (A) were measured as described in Experimental Procedures. Data are mean ± SD for seven mice per group.

(E) Serum chemistry. Serum levels of AST and ALT were determined as described in Experimental Procedures. Data are mean ± SD for seven mice per group and are representative of two independent experiments.

showed no defects in hematopoiesis or lymphopoiesis or in heart development.

Immune responses are coordinated by interactions within the cytokine-cytokine receptor network. $CD4^+$ T cells potentiate the inflammatory or humoral immune responses to bacterial, parasitic, and viral agents by differentiating into Th1 or Th2 effectors (Mosmann and Coffman, 1989; Reiner and Locksley, 1995; Mosmann and Sad, 1996). IL-12-induced production of IFN-γ by Th1 cells is critical for defense against intracellular mi-

per group and are representative of four independent experiments. *$p < 0.001$ as compared with WT cells treated with *L. major* antigen.

(D) RT-PCR analysis of cytokine mRNA expression in popliteal LN $CD4^+$ T cells isolated from BALB/c (B/c), $WSX\text{-}1^{+/+}$ (WT), and $WSX\text{-}1^{-/-}$ (KO) mice 2 weeks after *L. major* infection. β-actin, internal control. Experiments were repeated three times with similar results.

(E) Flow cytometric analysis of intracellular IFN-γ production 2 weeks after *L. major* infection. Popliteal LN cells from $WSX\text{-}1^{+/+}$ (WT) and $WSX\text{-}1^{-/-}$ (KO) mice were cultured with *L. major* antigen for 24 hr. Intracellular IFN-γ was detected as described in Experimental Procedures. The numbers in the upper right quadrants are the percentages of IFN-γ-positive cells within the $CD4^+$ T cell population. Experiments were repeated three times with similar results.

(F) IFN-γ production by popliteal LN $CD4^+$ T cells from $WSX\text{-}1^{+/+}$ (WT) and $WSX\text{-}1^{-/-}$ (KO) mice 4 weeks after *L. major* infection. $CD4^+$ T cells were cultured with (hatched columns) or without (open columns) *L. major* antigen (Ag), and IFN-γ production was measured as in (C). Data shown are mean ± SD of triplicate samples from four mice per group and are representative of three independent experiments.

(G) RT-PCR analysis of cytokine mRNA expression in popliteal LN $CD4^+$ T cells isolated from BALB/c (B/c), $WSX\text{-}1^{+/+}$ (WT; four mice), and $WSX\text{-}1^{-/-}$ mice (KO; four mice) 4 weeks after *L. major* infection. β-actin, internal control. Experiments were repeated twice with similar results.

(H) Time course analysis of cytokine expression. Competitive PCR analysis was performed to quantitatively determine IFN-γ, IL-4, and β-actin (control) levels. cDNAs were prepared from popliteal lymph node $CD4^+$ cells of $WSX\text{-}1^{+/+}$ (WT), $WSX\text{-}1^{-/-}$ (KO), and BALB/c (B/c) mice on day 5 and at 2, 4, and 6 weeks postinfection. Appropriate amounts (normalized to β-actin) of cDNAs were coamplified with 5-fold serially diluted competitive fragments. Smaller sized bands are PCR products derived from competitive fragments. Comp = decreasing concentrations of competitive fragments added as templates.

(I) Serum immunoglobulin levels in $WSX\text{-}1^{+/+}$ (open circles) and $WSX\text{-}1^{-/-}$ (closed circles) mice 9 weeks after *L. major* infection. Data shown are mean ± SD of triplicate samples from four mice per group for IgG1 (left), IgG2a (middle), and IgE (right). Similar results were obtained using sera from mice at 3 weeks postinfection.

croorganisms in mice and humans (Romani et al., 1997). *IL-12*$^{-/-}$ mice infected with *L. major* exhibited lesions that were larger and progressed further than those in a wild-type, resistant strain (Mattner et al., 1996). Moreover, *IL-12*$^{-/-}$ mice mounted a polarized Th2 response to the pathogen rather than a Th1 response. In the current study, WSX-1-deficient mice also showed susceptibility to *L. major* infection with deviation to a Th2 cytokine profile, indicating an important role for WSX-1 in early defense against this parasite. However, IFN-γ production in response to *L. major* antigen was restored to normal at 4 weeks postinfection in *WSX-1*$^{-/-}$ mice, a recovery not reported for *IL-12*$^{-/-}$ mice. Footpad swelling was also reduced in some infected *WSX-1*$^{-/-}$ mice, presumably reflecting the restored IFN-γ production. Thus, whereas IL-12/IL-12R interaction is absolutely required to maintain a Th1 response against *L. major* infection (Park et al., 2000), WSX-1 (and its unknown ligand) play a more limited role, being required only for the initial production of IFN-γ and induction of Th1 responses. We conclude that an absence of WSX-1 signaling has a significant impact at the early stages of an intracellular infection, but this impact is mitigated by IL-12R signaling at later phases.

Several lines of evidence have shown that the formation of antimycobacterial granulomas depends on IFN-γ production. A disseminated form of tuberculosis was seen in knockout mice deficient for either IFN-γ or the p40 subunit of IL-12 (Cooper et al., 1993; Flynn et al., 1993). The poorly demarcated granulomas in BCG-infected *WSX-1*$^{-/-}$ mice resemble the lung granulomas of *M. tuberculosis*-infected *IL-12p40*$^{-/-}$ mice (Cooper et al., 1997). This similarity in phenotype suggests that IFN-γ function is impaired in the microenvironment surrounding the granuloma in *WSX-1*$^{-/-}$ liver, consistent with the reduced IFN-γ production exhibited by isolated *WSX-1*$^{-/-}$ splenocytes on day 2 postinfection (Figure 5C). However, the deficit in splenocyte IFN-γ production is small compared with that observed during *L. major* infection, and IFN-γ levels are in fact restored to normal by day 7 postinfection. Therefore, the precise cause of the defect in granuloma formation in BCG-infected *WSX-1*$^{-/-}$ mice remains under investigation. It is possible that even a small decrease in IFN-γ production in *WSX-1*$^{-/-}$ mice could inhibit the expression of IFN-γ-dependent chemokines such as IP-10 and Mig (Sallusto et al., 1998), resulting in abnormal recruitment of cells required for granuloma formation. Alternatively, like other multifunctional cytokine receptors, WSX-1 may convey IFN-γ-independent signals during BCG infection that affect the migration and/or homing of cells required for granuloma formation. Although the loose accumulations of mononuclear cells in the granulomas of BCG-infected *WSX-1*$^{-/-}$ mice may not be sufficient to confine the bacteria within phagocytes, WSX-1 appears to be formally dispensable for controlling the BCG pathogen. Liver CFU counts and serum chemistry values associated with liver damage were comparable in *WSX-1*$^{-/-}$ and wild-type mice. We speculate that other bactericidal effectors such as IFN-γ induced by IL-12 and/or IL-18, IL-1, or TNF-α may be sufficient to control BCG infection in the absence of WSX-1 signaling.

The ligand for WSX-1/TCCR is currently unknown. The homology of WSX-1/TCCR to IL-12R and the phenotypes of the knockout mice described above suggest that a cytokine structurally and functionally related to IL-12 is the most probable candidate. One possibility for the WSX-1 ligand is IL-23, a cytokine composed of the p40 subunit of IL-12 plus p19, a novel component. IL-12-like biological functions have recently been reported for this cytokine (Oppmann et al., 2000). It is highly unlikely that IL-12 itself is the primary ligand for WSX-1. As also shown by Chen et al. (2000), we found that T cells from *WSX-1*$^{-/-}$ mice proliferated normally in response to exogenous IL-12 treatment. Consistent with this finding, we demonstrated that IFN-γ production by *WSX-1*$^{-/-}$ Th1 cells fully differentiated in vitro in the presence of IL-12 was normal. In contrast, Chen et al. (2000) reported that in vitro Th1 differentiation in response to IL-12 was impaired in cells from their TCCR/WSX-1-deficient mice. The reason for this difference is unknown, but, because our "primary stimulation" *WSX-1*$^{-/-}$ CD4^{+} T cells produced less IFN-γ than wild-type cells (Figure 3D), it is possible that slight differences in cellular activation status could account for the discrepancy. Differences in gene disruption strategies and/or the genetic background of the mice may also be relevant.

The impaired production of IFN-γ by "primary" *WSX-1*$^{-/-}$ T cells is intriguing. This finding suggests that WSX-1 is required for normal production of IFN-γ when naive T cells first encounter antigens and that this role is later overshadowed by that of IL-12R in fully activated and differentiated effector cells. This hypothesis is in line with the observation by Chen et al. (2000) that the expression of *WSX-1* is downregulated in activated Th1 and Th2 effectors and with the fact that IL-12R is highly expressed in activated Th1 cells (Szabo et al., 1997; Wu et al., 1997). The functions of WSX-1/TCCR in the context of the cytokine network will no doubt be clarified by identification of the ligand and coreceptors, if any, for this molecule.

Experimental Procedures

Cells

E14K embryonic stem cells from 129/Ola mice were maintained on a layer of mitomycin C-treated embryonic fibroblasts in Dulbecco's Modified Eagle's Medium, supplemented with leukemia inhibitory factor, 15% fetal calf serum, L-glutamine, and β-mercaptoethanol.

Generation of *WSX-1*$^{-/-}$ Mice

Fragments of the murine *WSX-1* gene were cloned from a 129/J bacterial artificial chromosome library using a PCR-amplified *WSX-1* cDNA probe. A targeting vector was designed to replace a genomic fragment containing an exon encoding a portion of the second fibronectin type III domain with a neomycin resistance cassette. The targeting vector was linearized with NotI and electroporated into E14K ES cells. After G418 selection (200 μg/ml) (GIBCO-BRL), homologous recombinants were identified by PCR using a specific primer pair (5'-CCA AGG TGT CTC AGG GTC TAA C-3' and 5'-GGA AGG GGC CAC CAA GAA CG-3'). Five clones heterozygous for the targeted mutation were injected into 3.5 day C57BL/6 blastocysts, which were subsequently transferred into pseudopregnant foster mothers. Chimeric mice were crossed with C57BL/6 mice to produce heterozygous *WSX-1*$^{+/-}$ mice. Germline transmission of the mutation was verified by PCR and Southern blot analysis of tail DNA. Heterozygotes were intercrossed to generate homozygous *WSX-1*$^{-/-}$ mice. Homozygous and heterozygous mutant mice were backcrossed into C57BL/6 more than nine times before use in experiments.

WSX-1 gene expression was examined by RT-PCR in wild-type

$CD4^+$, $CD8^+$, and $B220^+$ cells sorted using magnetic beads (MACS, Miltenyi Biotec) or in plastic-adherent splenic macrophages (Hamano et al., 1998). The specific primer pair used was 5′-CAA GAA GAG GTC CCG TGC TG-3′ and 5′-TTG AGC CCA GTC CAC CAC AT-3′. For immunoprecipitations and Western blots, splenocytes were lysed and precleared with Protein A/G (Amersham Pharmacia Biotech) in a 1:1 mixture. Lysates were then incubated for 2 hr at 4°C with Protein A/G and 0.2 μl crude anti-WSX-1 antiserum. The antiserum was obtained by immunizing New Zealand White rabbits with a peptide (H_2N-CPKASAPIYSGYEKHFLPTPEELGLLV-COOH) representing the 26 C-terminal amino acids of WSX-1 coupled to KLH through an additional N-terminal cysteine. Immunoprecipitates were subjected to SDS-PAGE, transferred to PVDF membrane, and identified with a 1:1000 dilution of the same rabbit anti-WSX-1 peptide antiserum plus HRP-conjugated Protein A (Amersham Pharmacia Biotech). The signal was visualized using ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Flow Cytometric Analysis

Mononuclear cells from thymus, spleen, and lymph nodes were obtained using standard methods. Single-cell suspensions of thymocytes from *WSX-1*$^{+/+}$ or *WSX-1*$^{-/-}$ mice were stained with phycoerythrin (PE)-conjugated anti-CD4 and fluorescein isothiocyanate (FITC)-conjugated anti-CD8 antibodies (PharMingen). Lymph node cells or splenocytes were stained with PE-conjugated anti-CD3 and FITC-conjugated anti-B220 antibodies (PharMingen). Cells were examined for the expression of surface markers using a flow cytometer (FACScalibur, Becton Dickinson).

T Cell Proliferation and Cell Cycle Assays

For T cell proliferation, spleen cells (2×10^5 cells/well) from either wild-type or WSX-1-deficient mice were stimulated for 72 hr in a 24-well plate with either 2.5 μg/ml ConA or 0.0001–1.0 μg/ml of plate-bound anti-CD3ε monoclonal antibody (145-2C11, PharMingen). To assess IL-12 responsiveness, cells were stimulated as above for 72 hr in the presence or absence of 1.0 or 10 ng/ml anti-CD28 antibody (37.51, PharMingen) and increasing doses of IL-12 (0–16,000 pg/ml) were added to the plates. Cell proliferation was measured with Alamar blue (BioSource International) as described elsewhere with minor modifications (Breinholt and Larsen, 1998). Briefly, Alamar blue was added after 72 hr of culture and the plates analyzed on a fluorescent microplate reader 4 hr later.

For cell cycle analysis, purified spleen T cells were stimulated as above with plate-bound anti-CD3 antibody (1 μg/ml) (PharMingen) plus soluble anti-CD28 antibody (1 μg/ml) (PharMingen) for 46 hr. Cells were then suspended in 1 ml hypotonic fluorochrome solution (propidium iodide; PI [50 μg/ml] in 0.1% sodium citrate plus 0.1% Triton X-100 with RNase [500 μg/ml]) as previously described (Yoshida et al., 1995). The PI fluorescence of individual nuclei was measured using a flow cytometer, and cell cycle analysis was performed with ModFit LT software (Becton Dickinson).

In Vitro Induction of T Cell Differentiation

In vitro differentiation of $CD4^+$ T cells into Th1 or Th2 subsets was performed as described previously (Yoshida et al., 1998; Chen et al., 2000). Briefly, for primary stimulations, $CD4^+$ T cells (1×10^6/ml) purified with magnetic beads (MACS) were activated in the presence of irradiated (30 Gy) syngeneic spleen cells (1×10^6/ml) and ConA (2.5 μg/ml). The culture medium was supplemented with IL-2 (20 U/ml) and either titrated doses of IL-12 (0–3.5 ng/ml) plus anti-IL-4 antibody (PharMingen, clone 11B11) (500 ng/ml) for Th1 induction, or IL-4 (1000 U/ml) for Th2 induction. For secondary stimulations, cells were washed 3 or 7 days after the primary stimulation, counted, and restimulated at 1×10^6/ml in the presence of ConA (2.5 μg/ml) without any additional cytokines for 24 hr. The supernatants from both primary and secondary stimulation cultures were collected and analyzed for the production of IFN-γ or IL-4 by ELISA using ELISA Development Kits (Genzyme) according to the manufacturer's directions.

L. major Infection and Cytokine Analyses

L. major (MHOM/SU/73-5-ASKH) were passaged in vivo and grown in vitro in Medium 119 with 10% heat-inactivated fetal bovine serum containing 2 mM glutamine, 10 mM HEPES, and gentamicin (100 μl/ml). For infection, mice were subcutaneously inoculated in the right hind footpad with 5×10^6 stationary phase promastigotes. The footpad lesion was monitored weekly with a vernier caliper and compared with the thickness of the uninfected left footpad.

For analysis of cytokine production, popliteal LN cells (5×10^5/200 μl/well) were stimulated with or without *L. major* antigens (equivalent to 5×10^5 promastigotes) in the presence of irradiated (30 Gy) splenocytes (5×10^5/200 μl/well) for 66 hr. Culture supernatants were collected and analyzed for IFN-γ by ELISA as above.

For RT-PCR analysis of cytokine expression, popliteal LN of mice infected with *L. major* were isolated, and $CD4^+$ T cells were purified using magnetic beads. Total RNA was prepared, and the expression level of β-actin was first evaluated as an internal control using serially diluted reverse-transcribed cDNA. The expression levels of IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-10, and IL-13 were then assessed using appropriate pairs of primers. Primer sequences were as follows: IL-3, 5′-GAA GTG GAT CCT GAG G AC AGA TAC G-3′ and 5′-GAC CAT GGG CCA TGA GGA ACA TTC-3′; IL-5, 5′-CTC TAG TAA GCC CAC TTC TA-3′ and 5′-TGA TAC CTG AAT AAC ATC CC-3′; IL-10, 5′-TAC CTG GTA GAA GTG ATG CC-3′ and 5′-CAT CAT GTA TGC TTC TAT GC-3′; IL-13, 5′-CTC CCT CTG ACC CTT AAG GAG-3′ and 5′-GAA GGG GCC GTG GCG AAA CAG-3′. Primer sequences for other cytokines are described elsewhere (He et al., 1995). For quantitative determination of cytokine expression, competitive PCR analysis was performed using fragments generated with the Competitive DNA Construction Kit (Takara Biomedicals, Tokyo, Japan) according to the manufacturer's directions. Again, the expression of β-actin was first measured as an internal control. An appropriate dilution of each cDNA sample was then coamplified with 5-fold serially diluted competitive fragments.

For flow cytometric analysis of intracellular IFN-γ production, popliteal LN cells were isolated and cultured (1×10^6/ml) for 24 hr with *L. major* antigens (equivalent to 2.5×10^6 promastigotes). GolgiStop (PharMingen) was added to the culture for the last 6 hr. Cells were then stained with PE-conjugated anti-CD4 antibody (PharMingen), and fixed and permeabilized with the Cytofix/Cytoperm Plus kit (PharMingen) according to the manufacturer's directions. Cells were stained with FITC-conjugated anti-IFN-γ antibody (PharMingen) to detect intracellular IFN-γ and analyzed for the percentage of IFN-γ-positive cells.

For serum immunoglobulin analysis, ELISA assays were performed using the following antibodies: rat anti-mouse IgG1 (Zymed: 04-6100) and rat anti-mouse IgG1-HRP (Biosource: AMI2311) for IgG1; goat anti-mouse IgG2a (BETHYL) and rabbit anti-mouse IgG2a-HRP (CAPPEL: 50283) for IgG2a; and rat anti-mouse IgE (MCA419) (Serotec: 240399) and rat anti-mouse IgE-biotin (MCA420B) (Serotec: 5090) for IgE.

BCG Infection

Mice were infected i.v. with 5×10^5 *M. bovis* BCG (Connaught Laboratories) and sacrificed on day 2, 4, 7, or 14 postinfection. Livers were isolated for histological examination and CFU count, and serum was collected for AST, ALT, and IFN-γ measurements. For histological examination, livers were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. BCG-induced granulomas were counted in livers in 10 randomly marked microscopic fields at a magnification of 200×. For CFU counts, livers were homogenized in saline to obtain an extract that was serially diluted in saline and plated on Middlebrook 7H11 agar (Acumedia). Colonies were counted after 2 weeks incubation at 37°C and the results expressed as CFU/g of liver. AST and ALT enzymatic activities were measured using commercially available kits following the manufacturer's instructions (Boehringer Mannheim). For IFN-γ production by spleen cells, splenocytes were stimulated with plate-bound anti-CD3 antibody (5 μg/ml) (PharMingen) and IL-12 (1 ng/ml) for 48 hr, and IFN-γ in the supernatants was assayed by ELISA as above. Results of all assays were expressed as the mean ± SD, and differences between groups were evaluated using the Student's t test.

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